The Drosophila Pkn protein kinase is a Rho/Rac effector target required for dorsal closure during embryogenesis

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The PKN family of PKC-related protein kinases constitutes the major Rho GTPase-associated protein kinase activities detected in mammalian tissues. However, the biological functions of these kinases are unknown. We have identified a closely related PKN homolog in Drosophila (Pkn) that binds specifically to GTP-activated Rho1 and Rac1 GTPases through distinct binding sites on Pkn. The interaction of Pkn with either of these GTPases results in increased kinase activity, suggesting that Pkn is a shared Rho/Rac effector target. Characterization of a loss-of-function mutant of Drosophila Pkn revealed that this kinase is required specifically for the epidermal cell shape changes during the morphogenetic process of dorsal closure of the developing embryo. Moreover, Pkn, as well as the Rho1 GTPase, mediate a pathway for cell shape changes in dorsal closure that is independent of the previously reported Rac GTPase-mediated Jun amino (N)-terminal kinase (JNK) cascade that regulates gene expression required for dorsal closure. Thus, it appears that distinct but coordinated Rho- and Rac-mediated signaling pathways regulate the cell shape changes required for dorsal closure and that Pkn provides a GTPase effector function for cell shape changes in vivo, which acts together with a Rac–JNK transcriptional pathway in the morphogenesis of the Drosophila embryo.

[Key Words: Pkn; Rho GTPase; Rac GTPase; dorsal closure; signal transduction; Drosophila]

Received December 4, 1998; revised version accepted March 8, 1999.

The Rho family proteins comprise a subgroup of the Ras superfamily of regulatory GTPases that are ubiquitously expressed and highly evolutionarily conserved. The Rho GTPases, which include the Rho, Rac, and Cdc42 proteins, mediate a variety of cellular functions including the regulation of actin reorganization in response to extracellular stimulation. Specifically, activated Rho promotes the assembly of focal adhesions and stress fibers [Ridley and Hall 1992], Rac stimulates the formation of lamellipodia [Ridley et al. 1992], and Cdc42 induces filopodia formation [Nobes and Hall 1995]. Thus, these proteins function as critical regulators of cell morphology, motility, and adhesion. In addition to their regulatory roles in remodeling the actin cytoskeleton, Rho GTPases perform crucial functions in a variety of cellular processes, including cell growth and cell cycle progression, intracellular membrane trafficking, cytokinesis, and transcriptional regulation [for review, see Van Aelst and D'Souza-Schorey 1997]. However, the organization of GTPase-mediated signaling pathways that control these numerous and diverse biological functions and the nature of signaling specificity are just beginning to be elucidated.

Like all members of the Ras family of small GTPases, the Rho GTPases function as molecular switches, cycling between inactive GDP-bound forms and active GTP-bound forms. In recent years, numerous potential effector targets of the Rho GTPases have been identified by virtue of their ability to bind to the Rho proteins in a GTP-dependent manner. While some of these effectors exhibit specificity in their ability to bind to a single member of the Rho family of proteins, others can bind to multiple different Rho GTPases. This ability may account, in part, for the observation that distinct members of the Rho family can mediate similar or overlapping cellular functions. Furthermore, different effectors for the same Rho protein can potentially bind to distinct regions of the GTPase, thereby contributing to the specificity of signals to downstream biological processes [Sahai et al. 1998].

Many of the Rho/Rac/Cdc42 effector targets are kinases—predominantly serine/threonine protein kinases. In most cases, these targets appear to be activated by direct binding to the active Rho GTPases and might thereby initiate a signaling cascade that involves selective substrate phosphorylation leading to specific biological responses. For example, GTP-bound Rho activates its effector target, Rho kinase [ROK], which phosphorylates the myosin-binding subunit of myosin phosphatase, leading to smooth muscle contraction and actin stress fiber formation [Kimura et al. 1996]. It is also
possible that these GTPase-kinase interactions serve to regulate the subcellular localization of kinase activity, as appears to be the case for the Ras–Raf interaction [Marais et al. 1995].

Among the putative Rho/Rac effector targets in mammals are the protein kinase N/protein kinase C-related kinase (PKN/PRK) family of serine/threonine kinases (Amano et al. 1996; Watanabe et al. 1996). PKN [also referred to as PRK1] and the closely related protein PRK2 [Quilliam et al. 1996; Vincent and Settleman 1997] together account for the vast majority of Rho-binding autokinase activity detected in most mammalian tissues [Vincent and Settleman 1997]. The carboxy-terminal catalytic domains of these kinases are highly homologous to the PKC family kinases, but they possess unique amino-terminal regulatory sequences including three leucine zipper-like repeats shown to be important for the interaction with the Rho GTPase [Flynn et al. 1998]. These proteins also interact detectably with the Rac GTPase [Vincent and Settleman 1997; Flynn et al. 1998], suggesting that they may be shared effector targets of the Rho and Rac GTPases. Despite the identification of closely related PKN homologs in several organisms [Mukai et al. 1995; Stapleton et al. 1998], the precise biological function of these putative Rho targets remains unknown.

The ability of the Rho GTPases to regulate cell morphology and motility suggests that these proteins and their associated signaling pathway components are likely to perform functions essential to the normal morphogenesis of developing multicellular organisms. Indeed, recent studies of Drosophila from a number of laboratories have revealed a role for the Rho family proteins in a variety of developmental processes, including gastrulation [Barrett et al. 1997; Hacker and Perrimon 1998], oogenesis [Murphy and Montell 1996], axon outgrowth [Luo et al. 1996; Lamoureux et al. 1997; Zipkin et al. 1997], tissue polarity [Strutt et al. 1997], and dorsal closure (DC; for review, see Noselli 1998). Interestingly, in DC, a role for Drosophila homologs of each of the Rho, Rac, and Cdc42 proteins has recently been identified. DC is a major morphogenetic event that takes place relatively late in embryogenesis, in which epidermal cells stretch along the dorsal–ventral axis such that the two lateral epidermal cell sheets slide over the underlying amnioserosa cells [an epithelial layer] and eventually meet at the dorsal midline to close the dorsal side of the embryo [Martinez-Arias 1993]. DC does not require new cell divisions, and appears to depend solely on dramatic cell shape changes within a subset of epidermal cells. These shape changes are initially restricted to two symmetric rows of epidermal cells, known as the leading edge (LE) cells, and are followed by the stretching of the more lateral epidermal cells, ultimately resulting in the meeting of the two rows of LE cells at the dorsal midline.

Three classes of genes have been implicated in DC; namely, the Rho family of GTPases, the c-Jun amino (N)-terminal kinase [JNK] cascade components, including the Decapentaplegic [Dpp] signaling pathway genes, and several membrane-associated proteins [review, see Noselli 1998]. The recently described Drosophila loss-of-function Rho1 mutant is defective for DC, and homozygous mutant embryos exhibit an obvious hole in the dorsal–anterior portion of the larval epidermis [Strutt et al. 1997]. Although Rac1 loss-of-function mutants have yet to be reported, overexpression of Rac1N17, a dominant-negative form of Rac1, in developing Drosophila embryos, results in a DC defect [Harden et al. 1995]. Similar results have also been reported for Cdc42N17 [Riesgo-Escovar et al. 1996]. It has been reported previously that in mammalian cells, Rac and Cdc42, but not Rho, function as upstream activators of the JNK cascade [Cosoo et al. 1995; Minden et al. 1995]. Interestingly, Drosophila homologs of the mammalian JNK pathway genes, hemipterous [hep], JNK [Glise et al. 1995], basket [JNK, Riesgo-Escovar et al. 1996; Sluss et al. 1996], Djun [Glise and Noselli 1997; Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997b], and kayak [Dfos; Riesgo-Escovar and Hafen 1997a; Zeitlinger et al. 1997] are all required for DC. Mutations in any of those genes result in a dorsal-open phenotype very similar to that seen in either the Rho1 mutants or embryos overexpressing dominant-negative Rac1 or Cdc42. Furthermore, disruption of the JNK pathway in hep mutants abolishes the expression of two independent downstream target genes of Djun, dpp and puckered [a MAP kinase phosphatase] [Martin-Blanco et al. 1998], which are also required for DC [Glise and Noselli 1997]. The hep mutation also blocks an increase in dpp expression in the LE cells induced by expression of activated Rac1V12 in those cells. These results have led to a model for DC in which Rac1 [and possibly Cdc42] signals through the JNK pathway to activate the expression in LE cells of Dpp, a secreted ligand of the TGF-β receptor, which in turn relays an instructive signal to initiate stretching of the more lateral epidermal cells (Glise and Noselli 1997; Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997b). The signaling role of Rho1 or downstream effector targets of Rho1 in this process is unknown.

Here, we report the molecular cloning and biological characterization of a Drosophila homolog of the mammalian PKN family kinases. Drosophila Pkn binds specifically to both Rho1 and Rac1 GTPases in a GTP-dependent manner, and its kinase activity is promoted by both interactions, suggesting that Rho1 and Rac1 GTPases can utilize Pkn as a downstream effector target. A loss-of-function mutation in the Drosophila Pkn gene leads specifically to a DC defect during embryogenesis. However, this Pkn-mediated DC pathway is independent of the Rac–JNK-Dpp pathway, but rather, appears to act coordinately with this pathway to regulate epidermal cell shape changes during morphogenesis of the Drosophila embryo.

Results

Molecular cloning of Drosophila Pkn

In an effort to decipher the biological function of PKN and its role in GTPase-mediated signaling, we cloned a
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Drosophila PKN homolog, Pkn, using degenerate oligonucleotide-mediated PCR. The predicted Drosophila Pkn protein sequence is closely related to both human PKN and PRK2 (60% overall identity to both) and has all of the conserved features found in other PKN family members (Fig. 1A), including the amino-terminal negative regulatory pseudosubstrate motif (Kitagawa et al. 1996), the three leucine zipper repeats (HR1a, HR1b, and HR1c) that mediate GTPase binding (Watanabe et al. 1996; Flynn et al. 1998), a central conserved region of unknown function found in the PKC-ε and PKC-η kinases (Palmer et al. 1995), and the carboxyl-terminal PKC-like kinase domain (Fig. 1B). Recently, a partial cDNA sequence was also reported that encodes what appears to be the kinase domain of the same Drosophila Pkn gene (Ueno et al. 1997).

The expression pattern of the Drosophila Pkn gene is highly dynamic during embryogenesis (Fig. 1C). An in situ hybridization analysis of wild-type embryos revealed that Pkn mRNA is abundant at the blastodermal stage, suggesting that it is maternally loaded. At stage 13, when DC is normally initiated, the most prominent expression is seen in the dorsal LE cells and in two pairs of discontinuous stripes on the epidermis of each segment. However, the expression becomes more restricted in later stages and can only be detected in the anterior and posterior spiracles, the pharynx, and the mouth tip at stage 16.

Drosophila Pkn binds to activated Rho1 and Rac1 GTPases

Previous biochemical studies of the human PKN family members, PKN and PRK2, revealed that these proteins can bind directly to the Rho and Rac GTPases in vitro. Moreover, the kinase activity of the PKN/PRK2 proteins is activated by severalfold upon interaction with activated forms of these GTPases. Therefore, we examined potential protein interactions between Drosophila Pkn and several of the Rho family GTPases. For this analysis, an amino-terminally epitope-tagged form of Drosophila Pkn was expressed in transfected BOSC cells, and cell lysates were subjected to binding assays with GST fusions of Drosophila Rho1, Rac1, Rac2, and Cdc42. GTPase-associated Pkn was then detected by immuno-

Figure 1. Predicted protein sequence and expression pattern of Drosophila Pkn. (A) Conserved motifs and domains of the Drosophila Pkn protein and related human PKN family kinases. Homology is displayed as percent identical/similar for each compared domain. (PS) Pseudosubstrate; (LZ) leucine zipper; (RBD) Rho-binding domain; (PKC) PKC-ε and PKC-η kinase homology. (B) Alignment of amino acid sequences of the domains conserved between Pkn and its human homologs. Identical amino acids are shaded black. (C) Expression pattern of Pkn determined by RNA in situ hybridization. Embryos at the indicated developmental stages were incubated with a digoxigenin-labeled antisense RNA probe corresponding to a 3-kb Pkn cDNA encoding the kinase domain and the 3'-untranslated region. A stage 5 control embryo was hybridized with the corresponding sense RNA, revealing the apparent maternal loading of Pkn mRNA. Arrowheads in the stage 13 embryo indicate enriched Pkn expression in the LE cells.
 blotting of the epitope tag. As shown, Pkn associates specifically with Rhô1, Rac1, and Rac2, but not detectably with Cdc42 or GST alone (Fig. 2A). In addition, the observed binding is strictly dependent on the activated GTPγS-bound form of Rhô and Rac.

By performing similar binding assays with the isolated amino-terminal and carboxy-terminal regions of Pkn, we determined that both Rhô1 and Rac1 bind Pkn through the amino-terminal region of the protein (Fig. 2B). Significantly, it appears that Rhô1 and Rac1 interact with Pkn through distinct binding sites. This interaction is indicated by the fact that Rhô1, but not Rac1, exhibits a highly preferential interaction with an apparent breakdown product of Pkn which, on the basis of its gel mobility, is expected to lack the kinase domain but maintain the amino-terminal putative Rhô/Rac-binding sites and pseudosubstrate domain (Fig. 2A). To confirm this expectation, we generated a mutated form of Pkn in which a highly conserved amino acid in the HR1a domain (glycine 58) was substituted with alanine, and tested this form of Pkn for Rhô1 and Rac1 binding. As shown (Fig. 2C), the mutated form of Pkn binds Rhô1 well, but does not interact detectably with Rac1, suggesting that Rhô and Rac may bind Pkn through different HR1 domains.

Figure 2. Pkn is an effector target of Rhô and Rac GTPases. (A) Lysates of BOSC cells transfected with Flag-tagged Pkn plasmid were incubated with GST-fusion proteins on sepharose beads (preloaded with either GDP or GTPγS). Proteins bound to the beads were analyzed by anti-Flag immunoblotting. [Lysate] 3% of the input for each binding assay. (B) Similar binding assays with either the Pkn amino-terminal HR1 domain [left], or the Pkn carboxy-terminal region lacking the HR1 domain [right] were carried out as described in A. Both proteins are Flag tagged. The distorted mobility of Pkn HR1 bands in B [left] is due to the comigration of large amounts of the GST-fusion proteins. (C) Binding assays with either the wild-type Pkn protein or the Pkn mutant with the G58A substitution were carried out as described in A. Both Pkn proteins are Flag tagged, and both GST–Rhô1 and GST–Rac1 were preloaded with GTPγS. (D) Lysates from Pkn-transfected cells were incubated either with GST-fusion proteins as described in Materials and Methods, or anti-Flag antibody and protein-G beads (immunoprecipitation) before the washed samples were split into two halves. One half was analyzed by SDS-PAGE and anti-Flag immunoblot. The other was subjected to an autokinase assay followed by SDS-PAGE and autoradiography. The amount of Flag–Pkn in the immunoblot corresponds to 1/16 of the total immunoprecipitated protein, while the radioactive band in the autoradiograph corresponds to 1/2 of the total immunoprecipitated protein. (E) Pkn kinase activation upon Rhô1 or Rac1 binding was quantitated by autoradiography, and the results from three independent experiments were averaged. Error bars represent standard errors of the mean [S.E.M.].
Pkn mutant embryos are defective for DC

The Pkn gene was mapped to the cytogenetic region 45C. Several previously described P-element insertions map to this same region, and we found that one of these is inserted in an intron whose 5’ end is located 21 bp downstream of the putative ATG start codon of the Pkn-coding sequence within the genomic DNA (Fig. 3A). Flies harboring this P-element insertion, when made homozygous, exhibit a multi-phasic lethal phenotype in which flies die from late embryonal through pupal stages, but no adult homozygous mutants emerge. Although homozygous mutant embryos do not exhibit obvious developmental defects prior to stage 13, ~10% of them die as embryos with an obvious hole in the anterior region of the dorsal epidermis (Fig. 3E). This dorsal-open phenotype closely resembles the DC defects observed previously with loss of function of the Rho1 gene (Fig. 3C) and several components of the Rac-mediated JNK cascade.

Two independent lines of evidence indicate that the observed lethality and dorsal-open phenotypes are caused by disruption of the Pkn gene by the P-element insertion. First, transposase-mediated precise excision of the P-element yields fully viable and fertile adults, and no dorsal-open embryos were observed among >450 relevant progeny examined (Table 1). Second, expression of a Pkn cDNA encoding the full-length Pkn protein under the control of a heat-shock promoter in transgenic flies can rescue the viability and fertility of P-element-harboring flies and significantly reduces the number of dorsal-open embryos (Table 1).

To examine the requirement for maternally loaded Pkn mRNA, germ-line clone (GLC) mutants of Pkn were generated and ~50% of the mutant embryos derived from these clones were used to display the same DC defect (Fig. 3F). Phenotypic analysis of these embryos with various histological markers revealed that they are correctly patterned and do not exhibit any detectable defects of the central or peripheral nervous system or the somatic musculature (data not shown), suggesting that Pkn is not

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DC embryos (%)</th>
<th>Total no.</th>
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<tbody>
<tr>
<td>Pkn+/Pkn</td>
<td>9.6 ± 1.6</td>
<td>1014</td>
</tr>
<tr>
<td>Pkn[87]/Pkn[56]</td>
<td>0</td>
<td>451</td>
</tr>
<tr>
<td>GLC/Pkn</td>
<td>55.2 ± 0.4</td>
<td>1066</td>
</tr>
<tr>
<td>GLC/Df(45C)</td>
<td>48.2 ± 2.4</td>
<td>617</td>
</tr>
<tr>
<td>GLC/Pkn; P[hs–Pkn]/+ (heat shock)</td>
<td>14.6 ± 3.2</td>
<td>1112</td>
</tr>
<tr>
<td>GLC/Pkn bsk</td>
<td>76.2 ± 5.6</td>
<td>2998</td>
</tr>
<tr>
<td>GLC/Pkn Rho172R</td>
<td>68.2 ± 1.2</td>
<td>2613</td>
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Crossovers were set to obtain embryos of the indicated genotypes. Except for the Pkn heat shock rescue cross (line 5), all crosses were maintained overnight at 25°C to collect 0- to 18-hr embryos, and the percentage of embryos with dorsal closure defects was determined as described. For the heat shock rescue experiment, a 0- to 2-hr collection of embryos was allowed to age for 5 more hours before a 30-min heat shock at 37°C was administered. Embryos were then treated as those of other groups. The percentages of embryos with DC defects as compared to the expected total number of mutants (or the total number of all fertilized embryos in the case of Pkn[56]) are listed. Results are shown as the means ± S.D. of several experiments, and n is the total number of genotypically relevant progeny examined.
required prior to DC. An identical dorsal-open phenotype, with similar penetrance, was observed when GLC females were crossed to males harboring a chromosomal deficiency uncovering the 45A-D region, which contains Pkn (Fig. 3G; Table 1). In addition, whole-mount embryo in situ hybridization with a Pkn probe revealed that the mRNA signal in the GLC-derived embryos was not detectable when compared to that of wild-type embryos [Fig. 3H–J]. Finally, in generating the precise P-element excisions described above, we isolated >30 independent deletion alleles (imprecise excisions), including a few that had several kilobases of flanking DNA deleted [but not the entire gene]. All of these alleles, when made homozygous, exhibit the same DC defect, with similar penetrance, as the original P-insertion allele, and none is mosaics, exhibiting the same DC defect, with similar penetrance, as the original P-insertion allele, and none is more severe. Together, these results suggest that the Pkn element mutant allele of Pkn is a genetic null allele and that Pkn normally plays a specific role in DC during Drosophila embryogenesis.

Pkn is not required for the Rac–JNK pathway in DC

According to the current working model of DC, the Rac1 and Cdc42 GTPases activate the downstream JNK cascade kinases to induce the expression of several genes in the LE cells, including dpp (Glise and Nozelli 1997; Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997b). Thus, dpp mRNA expression in the LE cells is a convenient assay of activation of the JNK cascade in vivo. Because Pkn functions biochemically as a Rac1 effector target, and mutations in Pkn cause a DC defect similar to that seen with JNK pathway mutants, we wanted to determine whether Pkn is required for the previously established Rac–JNK–Dpp pathway. Therefore, we examined the expression of dpp mRNA in Pkn mutant embryos. As reported previously, we confirmed that mutations in the JNK cascade gene hep (Glise et al. 1995) result in embryos that fail to express detectable levels of dpp mRNA in the LE cells [Fig. 4, cf. A with C, and B with D]. In contrast to the loss of dpp expression seen in hep mutant embryos, expression of dpp in the LE cells of Pkn mutants is not detectably affected [Fig. 4, cf. E and F with A and B], indicating that Pkn is not required for the previously reported Rac–JNK–Dpp pathway. This conclusion was also verified by examination of the expression of β-galactosidase from a puckered–lacZ enhancer trap, which has been shown previously to be eliminated in a hep mutant background (Glise et al. 1995). As shown, Pkn mutant embryos that exhibit an obvious dorsal-open phenotype retain normal levels of puckered–lacZ expression [Fig. 4O], confirming that the Rac–JNK pathway leading to transcriptional activation is not detectably affected by the absence of Pkn.

Next, we examined the shapes of epidermal cells of Pkn mutant embryos and found that all epidermal cells adopt an unstretched polygonal shape [Fig. 4L] following an initial apparently normal LE cell stretching [Fig. 4K], similar to that seen in the JNK pathway mutants, such as hep [Fig. 4J]. Thus, it appears that while both Rac–JNK- and Pkn-mediated signals are required for the stretching of epidermal cells required for DC, they are associated with distinct pathways. Because the expression of Pkn mRNA is enriched specifically in the LE cells prior to DC, we also tested the possibility that Pkn expression is

Figure 4. Pkn functions in a Dpp-independent signaling pathway for DC. (A–F) Whole-mount dpp in situ hybridizations of stage 11 (left) and stage 13 embryos (center), in wild-type embryos (A, B), hep1 mutants (C, D), and Pkn GLC mutant embryos (E, F). [Black arrows] LE cells where normal levels of dpp expression were observed; [white arrows] LE cells in the hep1 mutants in which dpp expression is diminished (C, stage 11) or absent (D, stage 13). [G–L] Anti-fas-III staining of the lateral epithelia of stage 13 (G, I, K) and stage 16 (H, J, L) embryos during DC, in wild-type embryos (G, H), hep1 mutants (I, J), and GLC/Pkn1 mutants (K, L). [Black arrowheads] Correctly stretched LE cells; [white arrowheads] polygonal shaped unstretched lateral cells in stage 13 embryos. The black arrow in L shows incorrectly localized fas-III signal in the dorsalmost side of the LE cells in homozygous Pkn mutant embryos. [M–O] Anti-β-galactosidase staining of the puckered–lacZ enhancer trap line embryos in wild-type (M), hep1 (N), and Pkn GLC mutant (O) backgrounds. [Black double arrows] LE of various embryos where β-galactosidase staining is seen normally in the wild-type background. [P] mRNA in situ hybridization with a Pkn probe revealed that Pkn expression is unaffected in the hep1 mutant background, indicating that Pkn expression is not under the transcriptional control of the JNK pathway. [Black arrow] Pkn signal in the LE cells.
regulated by the JNK pathway. However, we found that Pkn expression is unaffected in hep mutant embryos (Fig. 4P). Accumulating evidence suggests that the Rac1 GTPase, as well as some of the JNK pathway components, performs a function in the LE cells that may be distinct from the regulation of gene expression, but is necessary for the stretching of the LE cells (Riesgo-Escovar and Hafen 1997b). Because we observed that Pkn is a biochemical effector of the Rac1 GTPase that mediates DC, but is not required for dpp or puckered gene expression, we tested the possibility that the Pkn mutant interacts genetically with the JNK pathway component, basket. We found that removal of one copy of the basket gene from a Pkn mutant GLC background significantly increases the frequency of dorsal-open embryos (from 55% to 76%; Table 1), suggesting that both Pkn and JNK activities converge at some point to affect a related aspect of the DC process.

The Rho1 GTPase is required in the LE cells for the cell shape changes during DC

The Rho1 GTPase has also been implicated in DC (Strutt et al. 1997). Null alleles of Rho1 exhibit a DC defect that closely resembles that seen in Pkn mutants (Fig. 3C) although the relevant Rho1-mediated pathway in this process has not been established. Because the cell shape changes in the LE cells appear to initiate the DC process, and Pkn expression is enriched in the LE cells just prior to DC, we wanted to explore the possibility that a Rho1–Pkn signal mediates shape changes in those cells. First, we examined the requirement for Rho1 activity specifically in the LE cells. For this analysis, we took advantage of the GAL4–UAS system (Brand and Perrimon 1993) to selectively express a dominant-negative form of Rho1 (Rho1<sup>N19</sup>; Barrett et al. 1997) in the LE cells (Glise and Noselli 1997) of wild-type embryos. More than 60% of these embryos display a dorsal-open phenotype very similar to that seen in the Rho1, Pkn, and JNK pathway mutants (Fig. 5A). Similar to Rho1 [not shown] and Pkn mutants, but not to a hep mutant, embryos expressing Rho1<sup>N19</sup> in the LE cells exhibit normal levels of dpp mRNA expression in the LE cells (Fig. 5B). Moreover, all of the epidermal cells in these embryos ultimately adopt an unstretched polygonal shape following a normal initial LE cell stretching at early stage 13 (Fig. 5C). This result demonstrates clearly that expression of the dominant-negative Rho1 in the LE cells does not cause a dorsal-open phenotype by nonspecifically blocking the Rac–JNK–Dpp pathway and suggests that a Rho1-mediated second instructive signal is generated in the LE cells, which together with Dpp, is required for the stretching of the more lateral–ventral cells.

As described above, we observed that Pkn mutant embryos as well as embryos expressing Rho1<sup>N19</sup> in the LE cells exhibit a DC defect despite the absence of a detectable reduction in dpp expression in the LE cells. Taken together with the observations that Pkn expression is enriched in the LE cells prior to DC, and Pkn appears to function biochemically as a Rho1 effector, these results are consistent with a role for a Rho1–Pkn signal that contributes to the stretching of the LE cells. To establish a specific requirement for Pkn in the LE cells, a transgenic line was established in which a putative dominant-negative form of Pkn (kinase deficient) is expressed under the control of the UAS element. By crossing this line to a line harboring the LE–GAL4 driver, it was determined that a small percentage of embryos exhibit a DC defect that is indistinguishable from that seen in Pkn or Rho1 loss-of-function mutants (Fig. 5A). We also examined a potential genetic interaction between Rho1 and Pkn. In this analysis, we found that removal of one copy
of the Rho1 gene (a null allele) from the Pkn mutant GLC background results in an increase in the frequency of dorsal-open embryos from 55% to 68% [Table 1]. The fact that a reduction of Rho1 activity enhances the frequency of DC defects observed in a Pkn-null background suggests that Rho1 most likely interacts with at least one additional downstream target that is also required for DC. Consistent with this hypothesis, it was observed that heat shock-induced Pkn expression is not sufficient to rescue the Rho1 mutant DC defect [data not shown].

Discussion

Drosophila Pkn is a shared effector target of Rho and Rac GTPases

We have identified a closely related homolog of the mammalian PKN family of serine/threonine kinases in Drosophila. The structural and biochemical properties of Drosophila Pkn appear to have been well conserved. Previous studies of the human PKN/PRK1 protein indicated that PKN binds specifically to the activated Rho GTPase, although we have also observed a weak, but reproducible, interaction between human PKN and the activated Rac GTPase [Vincent and Settleman 1997]. Human PRK2, on the other hand, was found to bind to the RhoA GTPase in a nucleotide-independent manner and to Rac in a strictly GTP-dependent manner [Vincent and Settleman 1997]. Moreover, each of these interactions results in an increase in the catalytic activity of the associated kinase. Thus, these proteins may mediate distinct signals via the Rho/Rac GTPases and may perform distinct cellular functions.

Significantly, the Drosophila Pkn protein exhibits approximately equivalent GTP-dependent binding to both Rho and Rac GTPases, consistent with a role for Pkn as an effector target of both GTPases. Although both GTPases interact with the amino-terminal HR1-containing region of Pkn, it is clear that Rho1 exhibits a strong binding preference for a kinase-deleted form of Pkn. Most likely, the full-length Pkn protein, but not the truncated form, can assume a conformation in which the carboxy-terminal kinase domain folds back to interact with the amino-terminal pseudosubstrate domain, thereby influencing interactions with the neighboring HR1 GTPase-binding motifs, as demonstrated previously for human PKN [Kitagawa et al. 1996]. Thus, the interactions between Drosophila Pkn and Rho1 and Rac1 may be mediated by distinct HR1 repeats in Pkn. Possibly, the distinct complexes of Pkn with either Rho1 or Rac1 result in different subcellular localizations of the protein or different substrate specificities of the GTPase-bound kinase. Alternatively, it is possible that Pkn function is sensitive to the simultaneous interaction with Rho and Rac proteins, a scenario in which Pkn may serve to integrate Rho- and Rac-mediated signals. However, we did not observe a synergistic effect of activated Rho1 and Rac1 proteins on Pkn kinase activity when all three proteins were co-incubated [Y. Lu and J. Settleman, unpubl.].
mutant clones in the adult eye, and the loss-of-function Pkn mutant does not suppress developmental defects associated with overexpression of Rho1 or Rac1 in the developing fly eye [Y. Lu and J. Settleman, unpubl.]. Together with the observation that these GTPases appear to play a role in establishing normal ommatidial polarity [Strutt et al. 1997], these results suggest that it is unlikely that Pkn is mediating the tissue polarity functions of Rho and Rac in eye development. However, we cannot exclude a role for Pkn in additional morphogenetic processes that take place during larval development.

**Rho1 mediates a Dpp-independent instructive signal for DC**

Although the precise role of Pkn in DC is not clear, for the following reasons, it appears likely that Pkn is transducing a Rho-dependent signal in the LE cells: [1] Pkn expression is enriched in the LE cells of stage 13 embryos; [2] the activated Rho1 GTPase binds to and activates the Pkn kinase in vitro; [3] loss-of-function Rho1 and Pkn mutants exhibit a very similar dorsal-open phenotype that is associated with a defect in stretching of the LE cells; [4] in both Rho1 and Pkn mutant embryos, expression of dpp mRNA in the LE cells is apparently normal; and [5] expression of the Rho1N19 and the PknKD mutants specifically in the LE cells (where Pkn expression is highly enriched just prior to DC) results in dorsal-open phenotypes that are indistinguishable from the Pkn mutant phenotype.

The fact that expression of Rho1N19 specifically in the LE cells leads to a cell nonautonomous stretching defect in the more lateral epidermal cells [despite normal dpp expression in the LE cells], suggests that Rho1 mediates a second, Dpp-independent, instructive signal (Fig. 6). Possibly, this signal is also mediated by a Rho–Pkn interaction. However, the finding that removal of one copy of Rho1 from a Pkn mutant GLC background [a genetic null] increases the frequency of dorsal-open embryos suggests that Rho1 is probably performing an additional, Pkn-independent, function in DC. In support of this hypothesis, we found that the heat shock–Pkn transgenic construct is unable to rescue the DC defect in Rho1 mutant embryos [Y. Lu and J. Settleman, unpubl.]. Thus far, the Rho effector target that mediates the ability of Rho to regulate gene expression via activation of the serum response factor, a known downstream target of Rho activation in mammalian cells [Hill et al. 1995], is unknown. Hence, it is possible that this second instructive signal is induced by a Rho-mediated transcriptional pathway, which is clearly distinct from the Rac-mediated transcriptional pathway. It is worth noting that our results do not exclude the possibility that the cell shape changes associated with the lateral epidermal cells require an additional direct (cell-autonomous) role for the Rho–Pkn pathway in those cells as well. It is clear, however, that the role of Rho1 in the LE cells during DC is distinct from that of Rac1.

**Coordinated Rho and Rac activities are essential for DC**

In light of the fact that *Drosophila* Pkn interacts equally well with the activated Rac GTPase, it is possible that a Rac–Pkn interaction also contributes to DC. However, the lack of a Rac1 loss-of-function mutant in *Drosophila* makes it difficult to examine the specific role of that interaction. Because the JNK pathway mutants are also associated with a defect in stretching of the LE cells, it has been suggested that components of the JNK pathway may mediate a Rac-dependent cell stretching signal that is unrelated to transcriptional regulation [Fig. 6]. It is difficult to imagine how Pkn could transduce a signal from Rac to this JNK-mediated cell shape change pathway and yet not be required for the Rac–JNK transcriptional pathway. However, it is possible that Pkn can transmit a Rac signal independent of the JNK–Dpp pathway. Indeed, we have found recently that the *Drosophila* gene *Myoblast city*, which is required for DC [Erickson et al. 1997], encodes a Rac-specific activator that does not appear to regulate *dpp* expression [Nolan et al. 1998]. This observation suggests that Rac may perform multiple functions in dorsal closure.

Significantly, there does seem to be some cross-talk between the Pkn-mediated signaling pathway and the JNK pathway. We observed, in a genetic interaction experiment, that removal of one copy of *basket* from a Pkn mutant GLC background significantly increases the fre-
quency of dorsal-open embryos. This result suggests that some component of JNK cascade signaling is sensitive to the activity of Pkn. Taken together with the fact that Rho1 generates a JNK–Dpp independent signal in the LE cells that is required for DC, it is clear from these studies that distinct but coordinated signaling pathways mediated by the Rho and Rac GTPases within the LE cells are essential for normal DC, and that Pkn is a strong candidate for an effector that mediates signals downstream of both GTPases.

Materials and methods

Molecular cloning methods

A genomic DNA fragment containing sequences encoding part of the kinase domain of a Drosophila homolog of mammalian PKN was isolated by PCR with a Drosophila genomic phage library and the following degenerate oligonucleotides: 5'-GGNAG(G/C)GGNCA(A/T)TT(A/T)GGNAA(G/C)GT-3' and 5'-A(A/T)TT(A/T)TC(A/T)TCNTCNTNCC-3'. The PCR product was subcloned and used as a probe to screen a Drosophila eye imaginal disc cDNA library and an embryonic cDNA library. Several positive phage clones were purified. Their inserts were subcloned into pBluescript (Stratagene) and used to assemble a full-length Pkn cDNA, in which an in-frame stop codon was found 3 bp upstream of the putative ATG start codon. The assembled cDNA was sequenced on both strands by the Sequenase kit (US Biochemicals) according to the manufacturer's instructions.

Polytene chromosome in situ hybridization

A Pkn probe labeled by digoxigenin (Genius kit 1; Boehringer Mannheim) was generated by PCR with the above-mentioned degenerate oligonucleotides and the subcloned Pkn genomic DNA according to manufacturer's recommendation. Chromosomal squashes were performed as described [Ashburner 1989], and in situ hybridization on polytene chromosomes was performed as described previously [Hariharan et al. 1995]. Upon confirming the 45C map position of the Pkn locus, we found that a partial previously reported kinase sequence, designated Pk45C, is identical to Pkn [Kalderon and Rubin 1988].

RNA in situ hybridization

In situ mRNA hybridization was performed essentially as described [Van Vactor and Kopczynski 1998]. In cases where a specific cross generated embryos of different genotypes, an anti-β-galactosidase antibody (Promega) was also used to mark the embryos with the balancer chromosome that also contained a P-element insertion carrying the lacZ gene.

GTPase binding and kinase assays

A 3.6-kb cDNA encoding the full-length Drosophila Pkn protein was subcloned into the mammalian expression vector pCMV5–Flag such that Pkn with a Flag epitope at its amino terminus was expressed when transfected into BOSC cells by use of the standard calcium phosphate method. Cell extracts were prepared 48 hr post-transfection in lysis buffer [50 mM HEPES [pH 7.4], 150 mM NaCl, 1.5 mM MgCl₂, 5 mM EGTA, 10% glycerol, 1% Triton X-100] and were precleared twice for 30 min at 4°C with protein A-Sepharose beads before use in binding assays. A similar strategy was used to express the isolated Flag-tagged Pkn HR1 domain and the Flag-tagged Pkn protein without the amino-terminal HR1 domains. Site-directed mutagenesis was used to generate the G58A point mutation in the Pkn cDNA according to the manufacturer's suggestion (QuickChange, Stratagene). The following pairs of oligonucleotides were used in the reaction: 5'-CTGAAAGATCAAAGAG-GCCGGCCGAGAAGCTCCG-3', and 5'-GCCGAGCTTCTGC-GCCGCCCTTTTGTATTCAG-3'. The G58A mutant Pkn with a Flag tag was expressed in BOSC cells as described above. Drosophila Rho1, Rac1, Rac2, and Cdc42 cDNAs were subcloned into pGEX20 vectors and GST fusion proteins were prepared according to standard methods. Equal amounts of GST fusion proteins and GST [30–60 µg/assay] were incubated at 30°C for 30 min in 50 µl of nucleotide exchange buffer [50 mM HEPES [pH 7.0], 5 mM EDTA, 0.1 mM EGTA, 50 mM NaCl, 0.1 mM DTT] with 0.5 mM of GDP or GTPγS, and the reaction was terminated by addition of MgCl₂ to a final concentration of 20 mM. Then, precleared lysates were incubated with the GST-GTPases (GDP or GTPγS bound) at 4°C for 1 hr before the beads were washed three times in wash buffer [20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100] and analyzed by SDS-PAGE, followed by immunoblotting with anti-Flag M2 monoclonal antibody [Sigma].

For kinase assays, Flag–Pkn captured either by GST-fusion proteins or M2 monoclonal antibody (immunoprecipitation) as described above was washed three times in wash buffer and once in kinase buffer [20 mM HEPES [pH 7.5], 100 mM NaCl, 10 mM MgCl₂] before assay. Half of each sample was subjected to immunoblotting to determine the relative amount of Flag–Pkn in each sample, while the other half was incubated at room temperature for 20 min in 25 µl of kinase buffer containing 2 µCi of [γ-32P]ATP [NEN]. The autokinase reactions were terminated by washing three times in wash buffer and boiling in SDS-containing sample buffer. After SDS-PAGE analysis and autoradiography, bands corresponding to Pkn were cut from the dried gel, and radioactivity was determined in a scintillation counter. The normalized radioactivities of the bands were compared to calculate the degree of Pkn activation. To facilitate the determination of the amount of Flag–Pkn in each sample, two-fold serial dilutions of the immunoprecipitated samples were compared with the amount of Flag–Pkn isolated by GST–GTPase capture.

Cuticle preparations

Overnight collections of embryos from various crosses were allowed to age at 25°C for 36 hr before dechorionation in 50% bleach and devellination. Cuticle preparations were carried out as described previously [Nüsslein-Volhard et al. 1984]
Germline transformations and heat shock rescue

A full-length Pkn cDNA was subcloned into the pCaSpeR-hs vector, and transgenic flies harboring this cDNA construct (P[hs–Pkn]) were generated according to standard procedures. A transgenic fly line carrying P[hs–Pkn] on the third chromosome was used to generate flies of the following genotype: Pkn(+;CyO; P[hs–Pkn]). To test the heat-shock rescue of the lethality associated with the P-element insertion, the F1 progeny growing at 25°C were subjected to 30-min heat shock at 37°C twice a day from 24 hr to 14 days after egg laying (AEL). Approximately 20% of the mutants could be rescued to adulthood and were fertile. Similar results were obtained with a different transgenic fly line that had a second chromosomal insertion of the same P[hs–Pkn] transgene.

GLC analysis

GLC mutants of Pkn were generated by a previously described method [Chou et al. 1993] using the Pkn(+/+) allele. The genotype w/flip/Y, P[w+m]; FRT42B[G13]; P[w+m]; ovoD1[P507] were mated to virgin females of the genotype w; P[w+m]; FRT42B[G13] Pkn(+/+) and heat shocked for 1 hr at 37°C on days 4, 5, and 6, to induce recombination. The female F1 progeny with the genotype w/y w/flip P[w+m]; 42B[G13]; P[w+m]; ovoD1[P507] P[w+m]; hs FRT42B[G13] Pkn(+/+) in which GLCs had been generated were then mated to males of the genotype Pkn(+;CyO wg–lacZ, w/Y, Df(2R)W75-1, cn/CyO wg–lacZ (referred to as Df[45C]/CyO in text; stock number 80763A from the Umea Stock Center, Sweden), or bsk(+/+) Pkn(+/+) (CyO wg–lacZ (generated by recombinating bsk(+)/ and Pkn(+/+) alleles) for phenotypic analysis.

Genetics

The stock that harbors a P-element insertion in the Pkn locus was obtained from the Bloomington Stock Center, with the stock description #P2322 P[ry/+] = P[Z1] 1(2)06736/06736 cn[1]/CyO; ry[306]. Rho1 mutant allele Rho1720 was from M. Modlzik (EMBL, Heidelberg, Germany). The LE–GAL4 driver line was obtained from S. Noselli [Centre National de Recherché Scientifique, Toulouse, France]. The UAS–Rho1719 was provided by D. Montell [Johns Hopkins University, Baltimore, MD]. The basket(+/CyO, Pkn(+/+)CyO and various other stocks were from the Bloomington and Umea Stock Centers. Precise excision of the P-element was carried out according to standard procedures. Briefly, P-element harboring flies of the genotype Pkn(+;+)/(+;CyO, ry[306] were mated to transposase-containing flies of the genotype CyO/Sp; Sh, D2-3/TM6, Ubx. The male F1 progeny of the genotype Pkn(+;+)/(+;CyO, Sh, D2-3/ ry[306] were selected, and each was mated individually with CyO/Sp; ry[306] virgin females. Approximately 160 such crosses were performed, and one to eight F2 male progeny of the genotype Pkn(+;+)/(+;CyO, ry[306] from each cross, which potentially harbored the excision allele of the original P-element, were mated individually with the original P-element harboring flies of the genotype Pkn(+;+)/(+;CyO, ry[306]). Only viables with CyO(+)/ flies of the genotype Pkn(+;+)/(+;Pkn(+;+)) were retained, and stocks were established with the rosy-eyed Pkn(+;+)/CyO, ry[306] progeny from the same cross.

To determine the percentage of embryos with DC defects, GLC virgin females were mated with males of the following genotypes, respectively: Pkn(+;CyO wg–lacZ, Df[45C]/CyO wg–lacZ, Pkn(+/+)bsk(+/CyO wg–lacZ, Pkn(+/+) Rho1720/CyO wg–lacZ, and Pkn(+/+)CyO wg–lacZ, P[hs–Pkn]). In the case of zygotic mutants, Pkn(+;+)/(+;CyO wg–lacZ flies were used. Overnight collections of 0- to 18-hr embryos were obtained, and all embryos were allowed to develop 48 hr more before progeny were dechorionated and counted. Alternatively, to count only mutants, overnight collections of embryos were stained for β-galactosidase to distinguish mutants from heterozygotes harboring a lacZ gene on the balancer chromosome. Embryos beyond stage 13 and of mutant genotypes were counted.

For anti-β-galactosidase staining of pucker–lacZ enhancer trap in a Pkn GLC or hep(+), a mutant background, a double balanced stock of Pkn(+;CyO, was generated on the balancer chromosome. Embryos beyond stage 13 and of mutant genotypes were counted. The presence of the pucker–lacZ enhancer trap in a Pkn GLC(+;+) (TM3 balancer) was indicated by the absence of the frizz–lacZ (TM3 balancer).

Acknowledgments

We thank Marek Mlodzik for the Rho1 mutant stocks, Stephane Noselli for the LE–GAL4 stock, Denise Montell for the UAS–Rho1719 stock, Laurel Raftery for the pucker–lacZ enhancer trap line, Ernst Hafen for the dpp cDNA clone, the Bloomington and Umea Stock Centers for the basket(+/CyO, Pkn(+/+)CyO and various other stocks. We are also grateful to David Van Vactor, Liz Perkins, Laurel Raftery, Fangli Chen, and members of our laboratory for helpful suggestions, and Laurel Raftery, Iswar Hariharan, Katie Nolan, Sylvie Vincent, Rosemary Foster, and Kathy Barrett, for critical reading of the manuscript. This work was supported by an award to J.S. from the American Cancer Society.

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The *Drosophila* Pkn protein kinase is a Rho/Rac effector target required for dorsal closure during embryogenesis

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*Genes Dev.* 1999, 13:

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